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MOM1 mediates DNA-methylation-independent silencing of repetitive sequences in *Arabidopsis*

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The heterochromatic regions around centromeres of animal and plant chromosomes are composed of tandem repetitive sequences, interspersed with transposons and transposon derivatives. These sequences are largely transcriptionally silent and highly methylated, and are associated with specifically modified histones. Although embedded in heterochromatin, *Arabidopsis* 5S ribosomal RNA genes are among the most highly transcribed genes. However, some 5S genes are silenced, and we show here that this silencing can be suppressed by a reduction in CG methylation. Importantly, we show that mutation of *MORPHEUS' MOLECULE 1* (*MOM1*) releases 5S repeat silencing independently of chromatin properties, as illustrated by the absence of detectable alteration of DNA and histone H3 methylation patterns. MOM1 also prevents transcription of 180-bp satellite repeats and *106B* dispersed repeats but not of transposons. Our results provide evidence that transcription of densely methylated and highly repetitive heterochromatic sequences is controlled by two distinct epigenetic silencing pathways, one dependent on and the other independent of DNA methylation.

Keywords: 5S genes; centromeric repeats; DNA methylation; MOM1; silent chromatin

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INTRODUCTION

Except for the nucleolus-organizing regions, the heterochromatin of *Arabidopsis* is clustered around centromeres in strongly 4,6-diamidino-2-phenylindole-stained chromocentres and is predominantly composed of tandem repeat arrays interspersed with transposable elements and their truncated derivatives. The sequences of these chromocentres are mostly transcriptionally silent, and distinct patterns of covalent modifications affecting both DNA and histone proteins are associated with this repressive chromatin environment (reviewed by Fuchs *et al*, 2006).

In plants, DNA methylation patterns at CG sites are maintained by the mammalian Dnmt1 orthologue METHYLTRANSFERASE 1 (MET1), whereas CHROMOMETHYLASE 3 (CMT3) is the main methyltransferase that maintains non-CG methylation at the transposable elements and centromeric repeats (Finnegan & Kovac, 2000; Bartee *et al*, 2001; Lindroth *et al*, 2001; Tompa *et al*, 2002; Lippman *et al*, 2003; Tran *et al*, 2005). Plants deficient in *MET1* and *CMT3* show a release of silencing from loci located in the chromocentres (Steimer *et al*, 2000; Johnson *et al*, 2002; Lippman *et al*, 2003; May *et al*, 2005). Mutation of DECREASE IN DNA METHYLATION 1 (DDM1), a putative Switch/Sucrose non-fermenting (SWI/SNF2)-like chromatin-remodelling factor, also leads to reduced DNA methylation and the release of silencing at chromocentre sequences (Hirochika *et al*, 2000; Steimer *et al*, 2000; Singer *et al*, 2001; Johnson *et al*, 2002; Lippman *et al*, 2003; May *et al*, 2005). Previous studies have identified MORPHEUS' MOLECULE 1 (MOM1) as a component of a silencing mechanism independent of DNA methylation marks (Amedeo *et al*, 2000; Steimer *et al*, 2000; Mittelsten Scheid *et al*, 2002; Probst *et al*, 2003). In contrast with *ddm1*, *mom1* releases silencing of *TRANSCRIPTIONALLY SILENT INFORMATION* (*TSI*) repeats at the chromocentres, without altering their DNA methylation status (Steimer *et al*, 2000). No endogenous targets of MOM1 other than *TSI* have been identified so far.

The tandemly repeated 5S ribosomal RNA genes are exceptional chromocentre sequences owing to their high transcriptional activity, and understanding the regulation of 5S gene expression in its heterochromatic environment is of particular interest. Although most of the 5S genes are actively transcribed, some 5S genes are silenced in wild-type plants (Mathieu *et al*, 2003). We show here that the silencing of 5S repeats is controlled by DNA-methylation-dependent and by MOM1-mediated, DNA-methylation-independent mechanisms. The same is true for the centromeric 180-bp satellite and *106B* repeats, but not for transposable elements, which do not seem to be targeted by MOM1, irrespective of element type and genomic position. In conclusion, our results show that the MOM1-mediated, DNA-methylation-independent pathway contributes to the silencing of highly repetitive sequences.

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RESULTS AND DISCUSSION

Previously, we described the existence of 5S genes that are silenced in WT plants, which we named minor 5S genes (Mathieu *et al*, 2003). Mutations in *DDM1* lead to a release of silencing of these genes, producing transcripts that differ from the main 5S rRNA sequence by one or two base substitutions (Mathieu *et al*, 2003). However, because of the indirect effect of *ddm1* on DNA methylation, it remains unclear whether the *ddm1*-induced release of silencing is mediated by a loss of DNA methylation or by the accompanying changes in the chromatin structure. Here, we investigated 5S gene silencing in plants carrying mutations in the DNA methyltransferases MET1 and CMT3.

To detect the release of 5S repeat silencing, we designed 5S-specific primer pairs that cover the entire 5S repeat sequence and used these in reverse transcription–PCR (RT–PCR) experiments. One primer pair detected low levels of 5S transcripts of 140 and 210 nucleotides (nt) in wild-type Columbia plants (Fig 1A,B). Both 5S transcripts accumulated to a higher level in *ddm1* than in wild-type plants and were named 5S-140 and 5S-210, respectively. In wild-type plants of Landsberg *erecta* and Zürich ecotypes, only 5S-210 transcripts accumulated at low levels, showing that the presence of 5S-140 transcripts is polymorphic between *Arabidopsis* ecotypes (Fig 1B). The sequencing of RT–PCR products from wild-type Columbia and *ddm1* plants showed that the 5S-210 transcripts originate only from the transcriptionally active 5S-repeat clusters located on chromosomes 4 and 5 (supplementary Fig 1 online). Importantly, similarly to minor 5S rRNAs, the sequence of the 5S-210 transcripts was heterogeneous, specifically in *ddm1*, and frequently showed 1 or 2 nt substitutions compared with the main 5S rRNA sequence (supplementary Fig 1 online). The 5S-140 transcripts differed from the 5S-210 transcripts by a 70-nt deletion and probably originated from the shorter 5S repeats present only in Columbia. The presence of a high level of 5S-210 transcripts in *ddm1* relative to

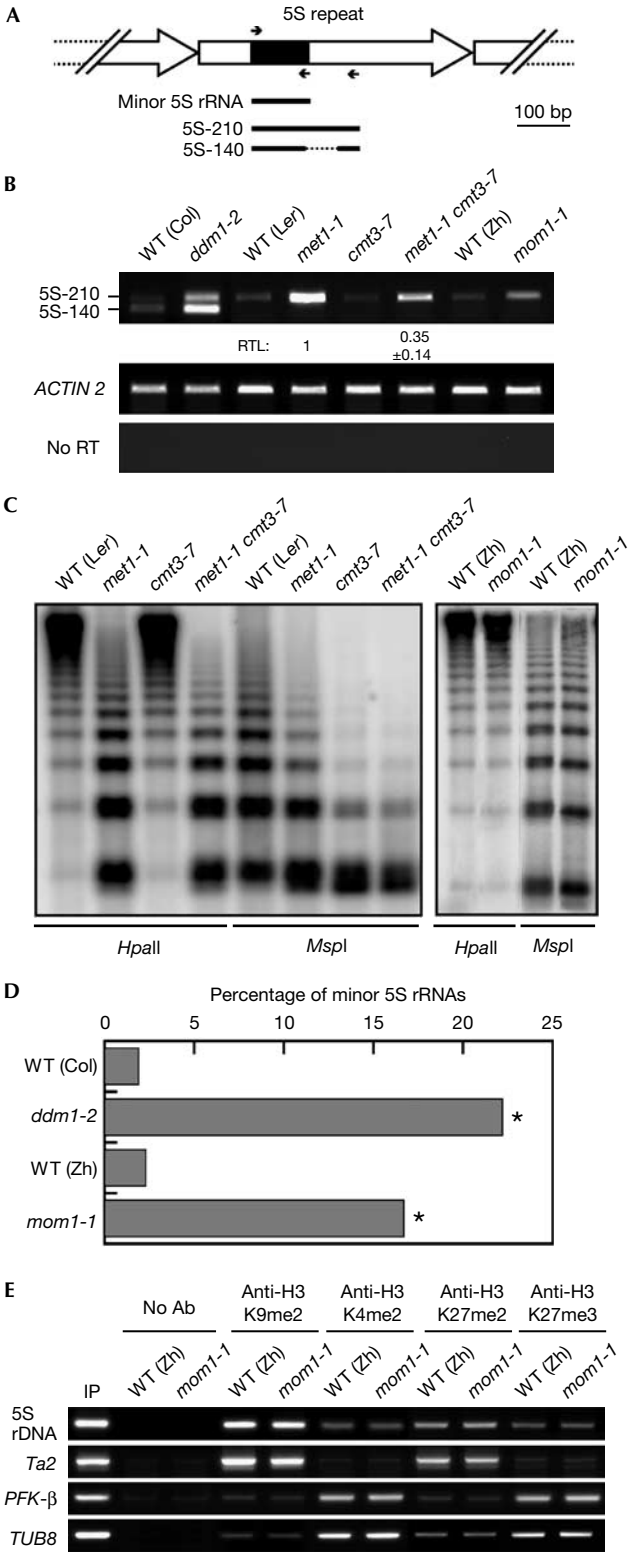

Fig 1 | Release of 5S gene silencing in silencing-deficient mutants. (**A**) A scheme of a 5S repeat, showing the position of the primers used in (**B**) and (**D**) and the regions being amplified. The region corresponding to 5S ribosomal RNA is represented by a black box. The dotted line indicates the 70-nucleotide deletion in 5S-140 relative to 5S-210. (**B**) Reverse transcription–PCR (RT–PCR) detection of 5S-210 and 5S-140 transcripts. Amplification of *ACTIN 2* was used to normalize the amounts of RNA template. Negative controls lacked reverse transcriptase (no RT). Numbers below the top gel indicate the relative RNA levels (RTL) of the 5S-210 transcript in *met1 cmt3* relative to *met1*, as determined by real-time RT–PCR. *met1-1*, *cmt3-7* and *met1-1 cmt3-7* are in the Landsberg *erecta* (L*er*) background; the *ddm1-2* and *mom1-1* mutations are in the Columbia (Col) and Zürich (Zh) backgrounds, respectively. WT, wild type. (**C**) DNA methylation of 5S genes. Genomic DNA was purified from the leaves of 3-week-old plants of the indicated genotype, digested with *Hpa*II or *Msp*I and probed with a 5S probe on DNA gel blots. (**D**) Proportion of minor 5S rRNAs recovered in *mom1-1* and *ddm1-2* (Mathieu *et al*, 2003) backgrounds. Asterisks denote significant differences compared with the WT values ($P<0.05$; Fisher's exact test). (**E**) Chromatin immunoprecipitation analysis of 5S rDNA using antibodies against H3K9me2 and H3K27me2, which are specific for heterochromatin, and against H3K4me2 and H3K27me3, which are specific for euchromatin. A heterochromatin control (the *Ta2* retrotransposon) and controls for euchromatin (the phosphofructokinase β-subunit (*PFK*-β) and the *TUBULIN 8* (*TUB8*) genes) are presented. Representative gel pictures of three independent replicates are shown. Ab, antibody; IP, input.

wild-type plants correlates with the release of silencing of minor 5S genes in this mutant (Mathieu *et al*, 2003). Therefore, we used the accumulation of 5S-210 transcripts as a marker of the release of silencing of 5S genes, rather than sequencing numerous 5S RT–PCR products to detect the presence of minor 5S RNAs.

The 5S-210 transcripts accumulated to a greater extent in *met1* but not in *cmt3* (Fig 1B). DNA gel-blot analysis of genomic DNA, after digestion with *Hpa*II (inhibited by methylation of either C in the sequence CCGG) and *Msp*I (inhibited by methylation of the outer C in the non-CG context CCGG), showed that the *met1* mutation strongly decreased CG methylation at 5S genes and, to a lesser extent, non-CG methylation; however, *cmt3* specifically reduced non-CG methylation (Fig 1C). The release of 5S gene silencing in *met1*, but not in *cmt3*, refines our previous conclusions from *ddm1* analysis, indicating that the silencing of 5S genes is controlled by MET1-mediated CG methylation, whereas non-CG methylation has little or no influence. Surprisingly, as confirmed by real-time RT–PCR, the *met1 cmt3* double mutant releases 5S gene silencing to a lesser extent than *met1* alone, although DNA methylation was reduced to a greater extent in the context of both CG and non-CG (Fig 1B,C; see the text below).

MOM1 is part of a silencing pathway that acts independently of DNA methylation (Mittelsten Scheid *et al*, 2002). We assessed whether this silencing mechanism also operates on 5S genes. The 5S-210 transcript level was higher in *mom1* than in wild-type plants; however, the dense cytosine methylation of the 5S genes remained unaffected (Fig 1B,C). The impact of *mom1* on 5S gene silencing was further confirmed by the higher proportion of minor 5S rRNA transcripts accumulating in *mom1* plants than in wild-type plants (Fig 1D). Importantly, chromatin immunoprecipitation (ChIP) analysis showed that the distribution of heterochromatin-specific (H3K9me2, H3K27me2) and euchromatin-specific (H3K4me2, H3K27me3) histone H3 marks at 5S rDNA was not modified in *mom1* plants in spite of the release of silencing (Fig 1E). The antibodies reacted as expected with the control sequences for heterochromatin (*Ta2* retrotransposon) and euchromatin (phosphofructokinase β-subunit (*PFK*-β) and *TUBULIN 8* (*TUB8*); Fig 1E). These results indicate that, in addition to a methylation-dependent silencing pathway involving *MET1*, 5S genes are also subjected to a MOM1-mediated silencing mechanism that acts independently of DNA and histone H3 methylation.

The unanticipated weak release of 5S gene silencing in *met1 cmt3* compared with *met1* plants motivated us to investigate the expression of *TSI*, another MOM1 target, in the *met1 cmt3* double-mutant background. DNA gel-blot analysis showed that DNA methylation at *TSI* and 5S genes is affected in an identical manner by *met1*, *cmt3* and *met1 cmt3* (Fig 1C; supplementary Fig 2 online). *TSI* transcripts of high molecular weight accumulated in *met1*, indicating that *TSI* silencing is strongly released owing to the loss of MET1 (Fig 2A). No *TSI* transcripts were detectable in *cmt3*, suggesting that, similar to 5S genes, a reduction in non-CG methylation is not sufficient to alleviate strongly the silencing of *TSI*. However, in contrast to the situation observed for 5S genes, the *TSI* transcript levels were higher in *met1 cmt3* than in either single mutant, indicating a synergistic effect of the mutations on the release of silencing at these repeats. This result indicates that CMT3-mediated non-CG methylation at *TSI* provides an additional epigenetic mark that reinforces MET1-mediated CG methylation and gene silencing.

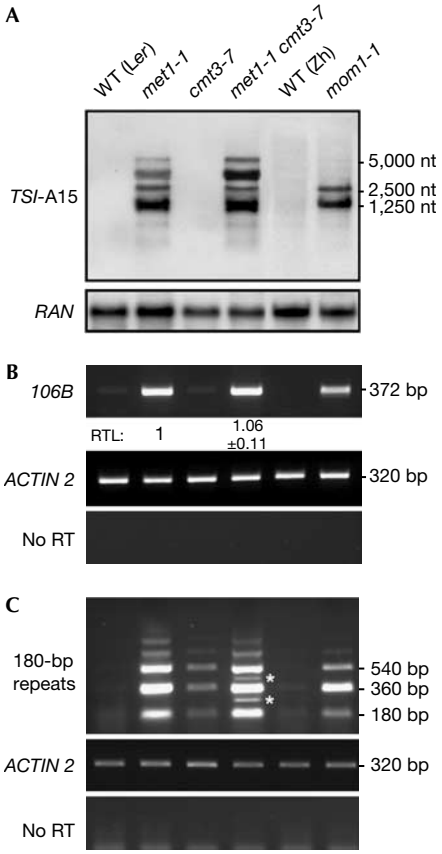

Fig 2 | Mutation of *MOM1* reactivates transcription of chromocentre repeats. (**A**) *TRANSCRIPTIONALLY SILENT INFORMATION* (*TSI*) transcript accumulation was analysed by RNA gel-blot analysis using the *TSI*-A15 probe (upper panel). The same blot was hybridized with a *RAN* probe as a loading control (bottom panel). Transcriptional analysis (**B**) of the *106B* LTR-like repeats and (**C**) of the 180-bp satellite repeats was performed by semiquantitative reverse transcription–PCR (RT–PCR). Numbers below the top gel in (**B**) indicate the relative RNA levels (RTL) of *106B* transcripts in *met1 cmt3* relative to *met1*, and were determined by real-time RT–PCR. Expression of *ACTIN 2* was used to normalize the amounts of RNA template. Negative controls lacked reverse transcriptase (no RT). The size of the amplicons is indicated on the right. Asterisks in (**C**) indicate the position of transcripts of intermediate size in *met1 cmt3*. Ler, Landsberg *erecta*; LTR, long terminal repeat; WT, wild type; Zh, Zürich.

Similar to our observation with 5S repeats (Fig 1E), Habu *et al* showed that H3 methylation patterns at *TSI* sequences are not affected by mutation of *MOM1*. These results contrast with the pronounced changes in histone H3 methylation patterns occurring in *met1* and *ddm1* at several heterochromatic sequences, including 5S and *TSI* repeats (Mathieu *et al*, 2003, 2005; Tariq *et al*, 2003). Moreover, nuclear architecture is altered in these two mutants, whereas *mom1* nuclei show a wild-type morphology (Soppe *et al*, 2002; Probst *et al*, 2003). This suggests that *mom1* alleviates silencing without altering chromatin properties.

In addition to the 5S genes and *TSI*, chromocentres contain large assemblies of 180-bp satellite repeats interspersed with *106B* long terminal repeat (LTR)-like dispersed repeats (Thompson *et al*, 1996; Fransz *et al*, 1998). Therefore, we investigated silencing at *106B* and 180-bp repeats in the *met1*, *cmt3*, *met1 cmt3* and *mom1* backgrounds. In agreement with previous data (May *et al*, 2005), RT–PCR assays detected a low level of *106B* transcripts in wild-type plants, which was strongly upregulated in *met1* (Fig 2B). The *106B* transcripts accumulated at similarly high levels in the *met1* and *met1 cmt3* backgrounds, as confirmed by real-time RT–PCR, whereas the *cmt3* mutant showed no increase in *106B* transcripts, in spite of a decrease in non-CG methylation (Fig 2B; supplementary Fig 2 online). This suggests that, similar to 5S genes, the silencing of *106B* repeats is mainly under the control of MET1-mediated CG methylation. RT–PCR with *mom1* RNA as a template showed upregulation of *106B* transcription relative to wild-type levels (Fig 2B), without a detectable alteration in DNA methylation patterns (supplementary Fig 2 online). Thus, *106B* repeats are also subjected to MOM1-mediated, methylation-independent silencing.

Elevated levels of 180-bp repeat transcript were also detected in *met1* and to a lesser extent in *cmt3*, confirming previous data (Fig 2C; May *et al*, 2005). Consistent with the impact of *cmt3*, we observed a synergistic effect of *met1* and *cmt3* mutations on 180-bp repeat transcription, as evidenced by the presence of transcripts of intermediate size in the *met1 cmt3* double mutant. In this respect, 180-bp repeats resemble *TSI*, with CMT3-mediated non-CG methylation and MET1-mediated CG methylation cooperating to establish full transcriptional silencing. Importantly, *mom1* also increases levels of 180-bp repeat transcripts (Fig 2C), without a detectable modification of DNA methylation status (supplementary Fig 2 online), showing that MOM1 controls the silencing of 180-bp repeats.

Chromocentres are enriched in transposable elements in addition to 5S genes, 180-bp satellite repeats, *106B* dispersed repeats and *TSI*. RT–PCR confirmed that two of these, the *Ta3* and *Athila* retroelements, were not reactivated in *mom1* plants (Fig 3), suggesting that LTR retrotransposons are not generally targeted by MOM1 (Steimer *et al*, 2000). Furthermore, we tested transcription of other transposable elements: two *Mutator*-like DNA transposons located either at the chromocentres (*MULE* At1g40097) or in a euchromatic environment (*MULE* At1g43280), the short interspersed nuclear element (SINE) *AtSN1* and the SINE-derived tandem repeats located upstream of the FWA gene (FWAtr), the last two residing in euchromatin. All but *MULE* At1g40097 were reactivated in the *met1* background (Fig 3), indicating that CG methylation suppresses transcription of different types of transposable element. Transcripts corresponding to *MULE* At1g40097 were detected only in *met1 cmt3*, and among all the transposons analysed, only *Ta3* and *AtSN1* transcripts were more abundant in the *met1 cmt3* than in the single-mutant backgrounds (Fig 3). As transposable elements of all types are predominant targets of CMT3 (Tran *et al*, 2005), it is likely that non-CG methylation at *MULE* At1g43280, *Athila* and *FWAtr* is also maintained by CMT3. In contrast with the methylation of CG dinucleotides, these observations indicate that CMT3-mediated non-CG methylation does not influence equally the silencing of all transposable elements. Importantly, none of the transposable elements assessed in the present study was reactivated in *mom1*

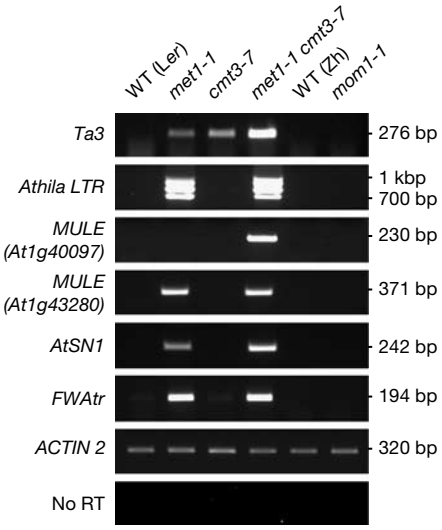

Fig 3 | Transcriptional analysis of various transposons. Semiquantitative reverse transcription–PCR was performed as described in Methods. Expression of *ACTIN 2* was used to normalize the amounts of RNA template. Negative controls lacked reverse transcriptase (no RT). The size of the amplicons is indicated on the right. L*er*, Landsberg *erecta*; LTR, long terminal repeat; WT, wild type; Zh, Zürich.

(Fig 3). Accordingly, Habu *et al* showed in an accompanying paper that the transposons *Mu1* and *Tar17* remain silent in *mom1*. Therefore, we conclude that transposable elements are not generally targeted by MOM1 for silencing, irrespective of their nature and genomic location. This also shows that MOM1 affects silencing at fewer loci than DNA methylation.

Among the targets analysed in the present study, the 5S genes were the only targets for which the release of silencing in *met1 cmt3* was weaker than in the *met1* single mutant (Fig 1B). Southern blot analysis indicated that the *MOM1* genomic sequence surrounding the promoter region is methylated in wild-type plants and loses some CG and non-CG methylation in *met1* and *met1 cmt3* and some non-CG methylation in *cmt3* (Fig 4A,B). RT–PCR and RNA gel-blot analyses showed that the *MOM1* transcript was slightly upregulated in *cmt3* and *met1 cmt3* but not in *met1* (Fig 4C,D), suggesting that *MOM1* transcription is influenced by non-CG methylation. Interestingly, this suggests that the MOM1-mediated silencing pathway is itself under the influence of DNA methylation, although MOM1 acts on its targets independently of DNA methylation. Given that MOM1 participates in the control of 5S gene silencing, we suggest that the upregulation of MOM1 in *met1 cmt3* might counteract the anticipated release of silencing induced by *met1* at 5S genes in the *met1 cmt3* double mutant. This implies that 5S genes show some as yet unknown specific features with respect to the other MOM1 targets.

Transposable elements seem to be targeted only by a methylation-dependent silencing mechanism in which MET1 has the main role. Although CMT3 preferentially targets transposable elements (Tompa *et al*, 2002; Tran *et al*, 2005), our results indicate that non-CG methylation does not influence equally the silencing of all transposons. Further studies are needed to clarify this issue.

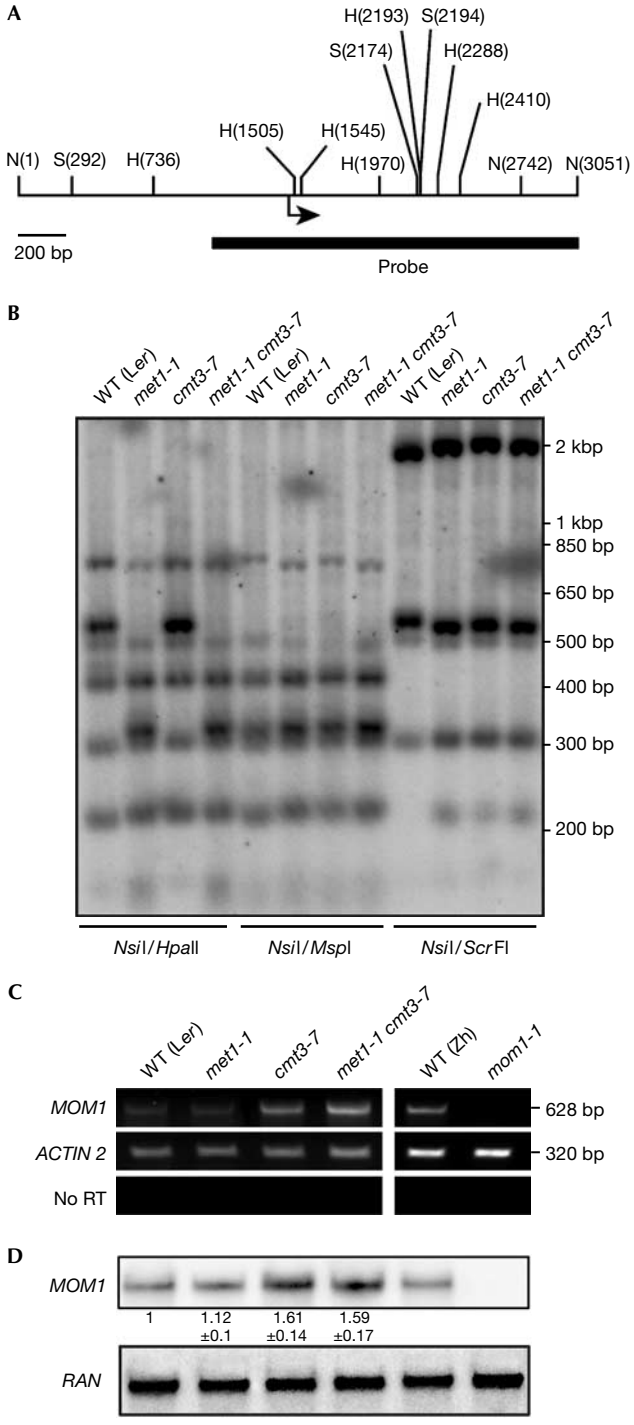

Fig 4 | Non-CG methylation influences *MOM1* transcription. (**A**) A schematic representation of the genomic region surrounding the *MORPHEUS' MOLECULE 1* (*MOM1*) gene transcription start site (hooked arrow). Recognition sites for the restriction enzymes are indicated with their position relative to the outermost *Nsi*I site. The region used as a probe in (**B**) is shown. N, *Nsi*I; H, *Hpa*II; S, *Scr*FI. (**B**) DNA methylation analysis of the promoter region of *MOM1*. Genomic DNA of the indicated genotypes was digested with *Nsi*I (methylation insensitive) followed by a secondary digestion with methylation-sensitive restriction endonucleases. The DNA gel blot was probed with a region surrounding the *MOM1* transcription start site. *Msp*I and *Scr*FI are sensitive to non-CG methylation, whereas *Hpa*II is sensitive to both CG and non-CG methylation. The position of size markers is indicated on the right. (**C**) Transcriptional analysis of *MOM1* by semiquantitative reverse transcription–PCR. Negative controls lacked reverse transcriptase (no RT). The size of the amplicons is indicated on the right. Expression of *ACTIN 2* was used to normalize the amounts of RNA template. (**D**) Northern blot analysis of the *MOM1* transcript. The same blot was hybridized with a *RAN* probe as a loading control (bottom). Quantifications of the *MOM1* transcript signal were performed on a phosphorimager (Molecular Imager FX; Bio-Rad, Hercules, CA, USA) and are indicated below the RNA blot. L*er*, Landsberg *erecta*; WT, wild type; Zh, Zürich.

Conversely, our data broaden the spectrum of MOM1 action and highlight that both methylation-dependent and MOM1 methylation-independent silencing pathways cooperate to silence densely methylated and highly repetitive heterochromatic sequences. As a next step, it is important to identify other components of the MOM1-mediated silencing pathway to gain insights into MOM1 target specificity and mode of action.

METHODS

Plant material. Plants were grown in soil in a greenhouse with an 18 h photoperiod at 22 °C. *ddm1-2* (Vongs *et al*, 1993) was in the Columbia background, *met1-1*, *cmt3-7* (Lindroth *et al*, 2001) and *met1-1 cmt3-7* (Johnson *et al*, 2002) strains were in the Landsberg *erecta* background and *mom1-1* (Amedeo *et al*, 2000) was in the Zürich background.

Gel-blot analysis. DNA gel-blot analyses were performed as described previously (Mathieu *et al*, 2003). Total RNA was extracted from leaf tissue using TRI reagent (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. For *TSI* RNA gel-blot analysis, 10 μg of total RNA per lane was used; for *MOM1* transcript analysis, we used 2–5 μg of polyA$^+$ RNA per lane. The different probes used are described in the supplementary information online.

Chromatin immunoprecipitation. ChIP was performed as described previously (Mathieu *et al*, 2005). The histone–DNA complexes were precipitated with antibodies against dimethyl H3K9, dimethyl H3K27, trimethyl H3K27 (Perez-Burgos *et al*, 2004) or dimethyl H3K4 (Upstate, Charlottesville, VA, USA). ChIP-PCR conditions are described in the supplementary information online.

RT–PCR analysis. Aliquots of 3 μg of total RNA were treated with RQ1-DNase (Promega, Madison, WI, USA) and 100 ng of DNase-treated total RNA was used as input in semiquantitative RT–PCR reactions using the OneStep RT–PCR kit (Qiagen, Valencia, CA, USA). Controls were without reverse transcriptase and were analysed to detect contaminating DNA. Amplification of *ACTIN 2* RNA was used as an internal control. Conditions for PCR and real-time RT–PCR are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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